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Regulation of the Potential Marker for Intestinal Cells, Bmi1, by β-Catenin and the Zinc Finger Protein KLF4

IMPLICATIONS FOR COLON CANCER[*] [S]

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Background: Bmi1 is a potential marker for the intestinal stem cells.
Results: Wnt regulates Bmi1 indirectly, while KLF4 directly inhibits Bmi1, as well as Bmi1-mediated histone ubiquitination in colon cancer cells.
Conclusion: Bmi1 is required for colon cancer cell proliferation, and it is up-regulated in colon cancer tissues.
Significance: Study of the mechanisms of Bmi1 regulation suggests potential targets for cancer therapeutics.

B lymphoma Mo-MLV insertion region 1 (Bmi1) is a Polycomb Group (PcG) protein important in gene silencing. It is a component of Polycomb Repressive Complex 1 (PRC1), which is required to maintain the transcriptionally repressive state of many genes. Bmi1 was initially identified as an oncogene that regulates cell proliferation and transformation, and is important in hematopoiesis and the development of nervous systems. Recently, it was reported that Bmi1 is a potential marker for intestinal stem cells. Because Wnt signaling plays a key role in intestinal stem cells, we analyzed the effects of Wnt signaling on Bmi1 expression. We found that Wnt signaling indeed regulates the expression of Bmi1 in colon cancer cells. In addition, the expression of Bmi1 in human colon cancers is significantly associated with nuclear β-catenin, a hallmark for the activated Wnt signaling. Krüppel-like factor 4 (KLF4) is a zinc finger protein highly expressed in the gut and skin. We recently found that KLF4 cross-talks with Wnt/β-catenin in regulating intestinal homeostasis. We demonstrated that KLF4 directly inhibits the expression of Bmi1 in colon cancer cells. We also found that Bmi1 regulates histone ubiquitination and is required for colon cancer proliferation *in vitro* and *in vivo*. Our findings further suggest that Bmi1 is an attractive target for cancer therapeutics.

Colorectal cancer is the third most commonly diagnosed cancer world-wide, maintaining a high death rate over the past ten years (1); thus great attention has been focused on mechanisms that lead to tumorigenesis in gastrointestinal epithelial cells. Aberration of the Wnt/β-catenin signaling pathway is one of the major causes of tumorigenesis (2, 3), yet it is not clear how the Wnt pathway cross-talks with other signaling pathways in intestinal homeostasis and cancer initiation. Krüppel-like factor 4 (KLF4),[2] a zinc finger protein highly expressed in the gut and skin, was recently found to interact with the β-catenin/TCF complex to repress Wnt signaling and inhibit tumor growth (4, 5). KLF4 is one of the four factors that induce pluripotent stem cells; thus playing a crucial role in stem cell regulation (6, 7). In a normal intestine, KLF4 inhibits proliferation of crypt progenitor cells and regulates the differentiation of goblet and Paneth cells (8, 9).

Intestinal stem cells are located in the bottom of crypts. Currently, the +4 label-retaining cells (LRC) model and the crypt base columnar cells (CBC) model suggest there are several stem cells per crypt to populate the entire crypt (10, 11). Because Wnt signaling plays essential roles in both normal intestinal stem cells and colon cancers, it was hypothesized that colon cancer is initiated from intestinal stem cells or progenitor cells (12). Lgr5 (or Grp49) is a leucine-rich repeat-containing G protein-coupled receptor; it is a Wnt target gene as well as an intestinal stem cell marker specific for CBC (13). In Wnt signaling-induced adenomas, the expression of Lgr5-EGFP was restricted to a small population of cells, suggesting that stem cells or progenitor cells are maintained in these tumors, supporting the cancer stem cell concept in colorectal tumorigenesis (14).

Another potential stem cell marker, B lymphoma Mo-MLV insertion region 1 (Bmi1), belongs to the polycomb group (PcG) gene family, which functions in gene silencing through chromatin modifications. Bmi1 is predominantly expressed in the +4 cells in the crypt (15). Bmi1 was initially identified as an oncogene that regulates cell proliferation and transformation (16,

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[2] The abbreviations used are: KLF4, Krüppel-like factor 4; Bmi1, B lymphoma Mo-MLV insertion region 1; PcG, Polycomb Group; PRC1, Polycomb Repressive Complex 1; CBC, crypt base columnar; HE, hematoxylin and eosin; AB, Alcian Blue; IHC, immunohistochemistry; TMA, tissue microarrays; Dox, doxycycline; dnTCF, dominant-negative TCF; CRC, colorectal cancer.

17). It was later found to play an important role in hematopoiesis and development of the nervous system (18). Bmi1 is also crucial for self-renewal of stem cells and cancer initiation (15, 19–21). The role of Bmi1 in controlling cell proliferation and self-renewal might be through its function as a polycomb group (PcG) protein, which facilitates histone modification and regulates gene silencing (22–24).

To get a deeper insight into the function and regulation of these stem cell markers, we analyzed the effects of Wnt signaling and KLF4 on the expression of Bmi1. We found that Wnt signaling enhances while KLF4 inhibits the expression of Bmi1. Bmi1 is required for colon cancer cell proliferation, and it is up-regulated in primary human colon cancers. The mechanisms of Bmi1 function and regulation in colon cancer were examined in this study.

EXPERIMENTAL PROCEDURES

Cell Lines, Lentiviral Transduction, Proliferation Tumor Xenograft Assay, Hematoxylin and Eosin (HE), and Alcian Blue Staining—LS174T colon cancer cell line (5) was grown in RPMI medium (Mediatech) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. The stable cell line LS174T-KLF4 has been described previously (5).

Bmi1 cDNA (Origene, CS116894) was cloned into lentivirus vector pCS-CG-hPGK (kindly provided by Dr. Tianyan Gao). Control shRNA and Bmi1 shRNA cloned in pLKO.1 vectors with puromycin resistance selection marker were from Sigma. Lentiviral stocks were prepared as previously described (25). LS174T cells were infected with lentivirus carrying pCS-CG-hPGK control vector and pCS-CG-hPGK-Flag-Bmi1, respectively; LS174T and/or HT29 cell lines were infected with lentivirus carrying pLKO.1-control shRNA and pLKO.1-Bmi1 shRNA, respectively. For the proliferation assay, stable cell lines were seeded as 2.5×10^4/well in 12-well plates and counted at appropriate times using the cell viability analyzer (Beckman Coulter, Vi-Cell XR).

For xenograft assay, HT29 stable cell lines (1×10^6) were injected subcutaneously into both flanks of athymic nude mice as described previously (26, 27). Tumor growth was analyzed twice weekly. Tumor xenografts were harvested and embedded in paraffin after 3 weeks. Alcian Blue (AB) staining was performed based on standard protocol using Alcian Blue 8GX and Fast Red from Sigma (kindly provided by Dr. Tianyan Gao). Hematoxylin and Eosin (HE) staining was performed by the Histology Laboratory of the Imaging Facility at University of Kentucky.

Western Blotting and Histone Extraction—Cells were lysed in appropriate volume of lysis buffer (50 mm HEPES, 100 mm NaCl, 2 mm EDTA, 1% glycerol, 50 mm NaF, 1 mm Na_3VO_4, 1% Triton X-100, with protease inhibitors). For histone extraction, cell pellet from lysate residues were resuspended with 0.2 N HCl and shaken at 4 °C overnight. The extract after centrifuge contained histones to be tested. The following antibodies were used: mouse anti-β-actin (Sigma, A1978), mouse anti-Flag (Sigma, F1804), mouse anti-Bmi1 (Millipore, 05–637), mouse anti-β-tubulin (Developmental Studies Hybridoma Bank, E7), and rabbit anti-uH2A (Millipore, 05-678).

Semi-quantitative RT-PCR and Quantitative Real-time PCR—LS174T-KLF4 cells were plated at $\sim 2 \times 10^5$ cells per well in a 6-well plate. The following day, doxycycline (1 μg/ml) was added to the culture medium. After 48 h of incubation, RNA was isolated using the RNeasy kit (Qiagen). Reverse transcriptase PCR (RT-PCR) was performed as described previously (5). The following primers were used: β -actin, 5′-CAACCGCGAGAAGATGAC-3′ and 5′-AGGAAGGCTGGAAGAGTG-3′; *CTNNB1*: 5′-TCTGTGTTGTTTTATGCCAT-3′ and 5′-CCATCCCTTCCTGTTTAGT-3′; *LGR5*: 5′-CCTGCTTGACTTTGAGGAAGAC-3′ and 5′-ATGTTCACTGCTGCGATGAC-3′; *BMI1*: 5′-AGCAGAAATGCATCGAACAA-3′ and 5′-CCTAACCAGATGAAGTTGCTG-3′; *RING1A*: 5′-CCATCAAGACCGAGTGCTTA-3′ and 5′-ACATCCTTCTCCATCCCCTTC-3′; *RING1B*: 5′-CAATGGCAATTGATCCAGTA-3′ and 5′-TGGTTTGATTCACCTTTGCT-3′.

Real-time PCR was performed according to standard protocols using TaqMan Gene Expression Assays (Applied Biosystems) including control eukaryotic 18 S rRNA (Hs99999901_s1) and Bmi1 (Hs00180411_m1).

Interference RNA, ChIP Assay, Luciferase Reporter Assay, and Immunohistochemistry (IHC) Staining on TMA Slides—Interference RNA, ChIP assay, luciferase reporter assay, and IHC were tested as described previously (5, 28).

Skin cancer tissue array from Biomax (CO482, Rockville, MD) was deparaffinized and IHC performed using standard protocols.

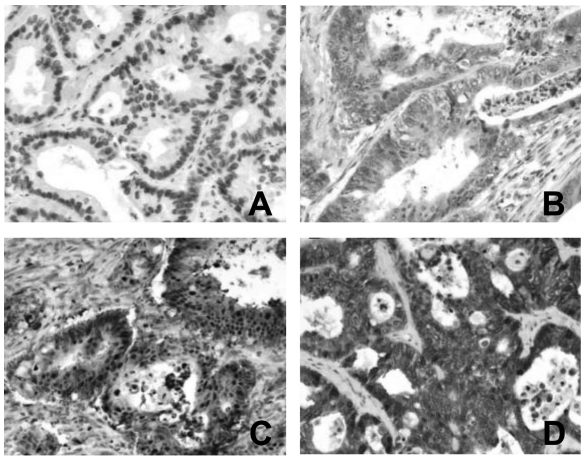
For the ChIP assay, the following primers were used: cyclin B1: 5′-TCTTGCCCGGCTAACCTTTCCAGG-3′ and 5′-TTCCGCCGCAGCACGCCGAGAAGA-3′; Bmi1: 5′-GGCGGCCGGGAGAAAGAAAGAAC-3′ and 5′-AGGGCGGCCTGGGAATTAGTGTC-3′.

For IHC staining, the following antibodies were used: KLF4 (Zhang *et al.*, 5), mouse anti-Bmi1 (Millipore, 05–637), and rabbit anti-β-catenin (Sigma, c2206).

Statistical Analysis—Descriptive statistics were calculated and bar graphs and line plots were generated to summarize cell proliferation, luciferase intensity qRT-PCR, tumor volume across cell culture conditions and between xenograft groups. A two-sample t test was employed to compare luciferase levels and analysis of variance for comparison of cell proliferation between shRNA groups and days of measurement. Linear mixed models were employed for comparison of tumor growth rate over time between control *versus* shRNA groups. Finally, analyses of IHC total scores (sum of intensity and percent staining) on colorectal cancer tissue specimens included calculation of Spearman correlation coefficient to assess correlations between Bmi1, KLF4, and β-catenin and nonparametric tests for comparison across grade and stages of colorectal tumors.

RESULTS

Bmi1 Is Overexpressed in Colon Cancer Tissues—Wnt/β-catenin plays a central role in normal intestinal development; deregulation of Wnt signaling leads to colon cancer. Wnt signaling regulates the self-renewal of intestinal stem cells and may also regulate the colon cancer stem cells. Lgr5, one of the potential stem cell markers, is specifically expressed in the crypt base columnar (CBC) cells in the intestine and has been identi-



E

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations		
	TOTB_CAT	**TOTBMI**
TOTB_CAT	1.00000 23	0.51825 0.0113 23
TOTBMI	0.51825 0.0113 23	1.00000 23

FIGURE 1. **Immunostaining of Bmi1 and β-catenin in colon cancer TMA.** *A*, colorectal cancer (*CRC*) with strong nuclear immunoreactions of Bmi1 (immunoreactivity score of 6 = percentage score of 3 and intensity score of 3). *B*, CRC with no immunoreactions of Bmi1 (immunoreactivity score of 0 = percentage score of 0 and intensity score of 0). *C*, CRC with strong nuclear localization of β-catenin. *D*, CRC with strong cytoplasmic immunoreaction but no nuclear localization of β-catenin. *E*, statistical analysis of correlation.

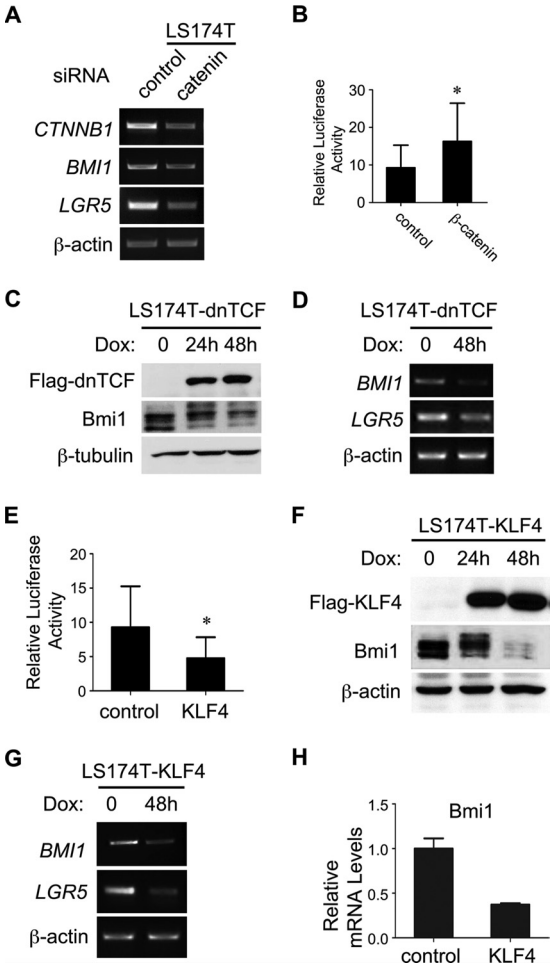


FIGURE 2. **Bmi1 is regulated by Wnt/β-catenin and KLF4.** *A*, semi-quantitative RT-PCR of Wnt target genes in LS174T colon cancer cells after transfected with control/β-catenin siRNA oligos. *B*, luciferase reporter assay testing the effect of β-catenin on Bmi1 promoter activity (*, $p = 0.0306$). *C* and *D*, Western blot and semi-quantitative RT-PCR showing expression of Bmi1 and Lgr5 in LS174T cells after induction of dominant negative TCF (*dnTCF*) in doxycycline (*Dox*)-inducible cells. *E*, luciferase reporter assay testing the effect of KLF4 on Bmi1 promoter activity (*, $p = 0.0228$). *F*, Western blot showing expression of Bmi1 in LS174T cells expressing Dox-inducible KLF4. *G*, semi-quantitative RT-PCR testing the transcription of Bmi1 and Lgr5 in LS174T cells after induction of KLF4 in Dox-inducible cells. *H*, real-time RT-PCR indicating mRNA level of Bmi1 expression under the effect of Dox-inducible KLF4 expression.

fied as a target of Wnt signaling (13, 29). Bmi1 is a novel stem cell marker expressed in the +4 cell at the bottom of crypt. To determine whether the expression pattern of Bmi1 correlates with β-catenin during colorectal progression, we stained tissue microarrays (TMA) with antibodies against Bmi1, β-catenin, and KLF4 (Fig. 1). TMA slides contained normal tissue samples and three stages of colorectal tumor tissue samples. We analyzed duplicated cores per case, 20 cases of colonic carcinoma and four cases of colonic normal tissue from necroscopy. Evaluation of the staining was based on the percentage of positive cells (nuclear staining) in each tissue core as well as intensity of the positively stained cells.

Both β-catenin expression and Bmi1 expression are significantly higher in colon cancer tissues than normal tissues. We found a positive correlation between nuclear β-catenin and Bmi1 in all tissue cores (Spearman Correlation Coefficient = 0.51825, $p = 0.0113$) (Fig. 1*E*, supplemental Tables S1 and S2).

Wnt/β-catenin Signaling Regulates Bmi1 Expression in Colon Cancer Cells—To test whether Bmi1 is a target of Wnt/β-catenin signaling, we treated LS174T colon cancer cells with β-catenin siRNA and analyzed the expression levels of Bmi1. We found that knockdown of β-catenin by siRNA decreased the levels of Bmi1 mRNA (Fig. 2*A*). As expected, β-catenin siRNA also inhibited the transcription of known Wnt target, Lgr5. To confirm this result, we performed a reporter assay using a luciferase gene driven by Bmi1 promoter (30). Overexpression of β-catenin increased the Bmi1 promoter activity (Fig. 2*B*).

We generated a stable colon cancer cell line that contains a doxycycline (Dox) inducible dnTCF (dominant-negative TCF), which inhibits wild type TCF function and attenuates Wnt signaling (5). We found that expression of dnTCF in LS174T colon cancer cells inhibited the expression of Bmi1 at both protein levels (Fig. 2*C*) and mRNA levels (Fig. 2*D*). This suggests the Wnt/β-catenin signaling regulates Bmi1 expression through a TCF/LEF-dependent mechanism. TCF/LEF binds a specific sequence in the promoter of its direct target (31); however, we could not find a consensus TCF/LEF binding site in the promoter region of Bmi, suggesting that Wnt/β-catenin signaling may regulate Bmi1 gene indirectly, probably through other β-catenin target genes.

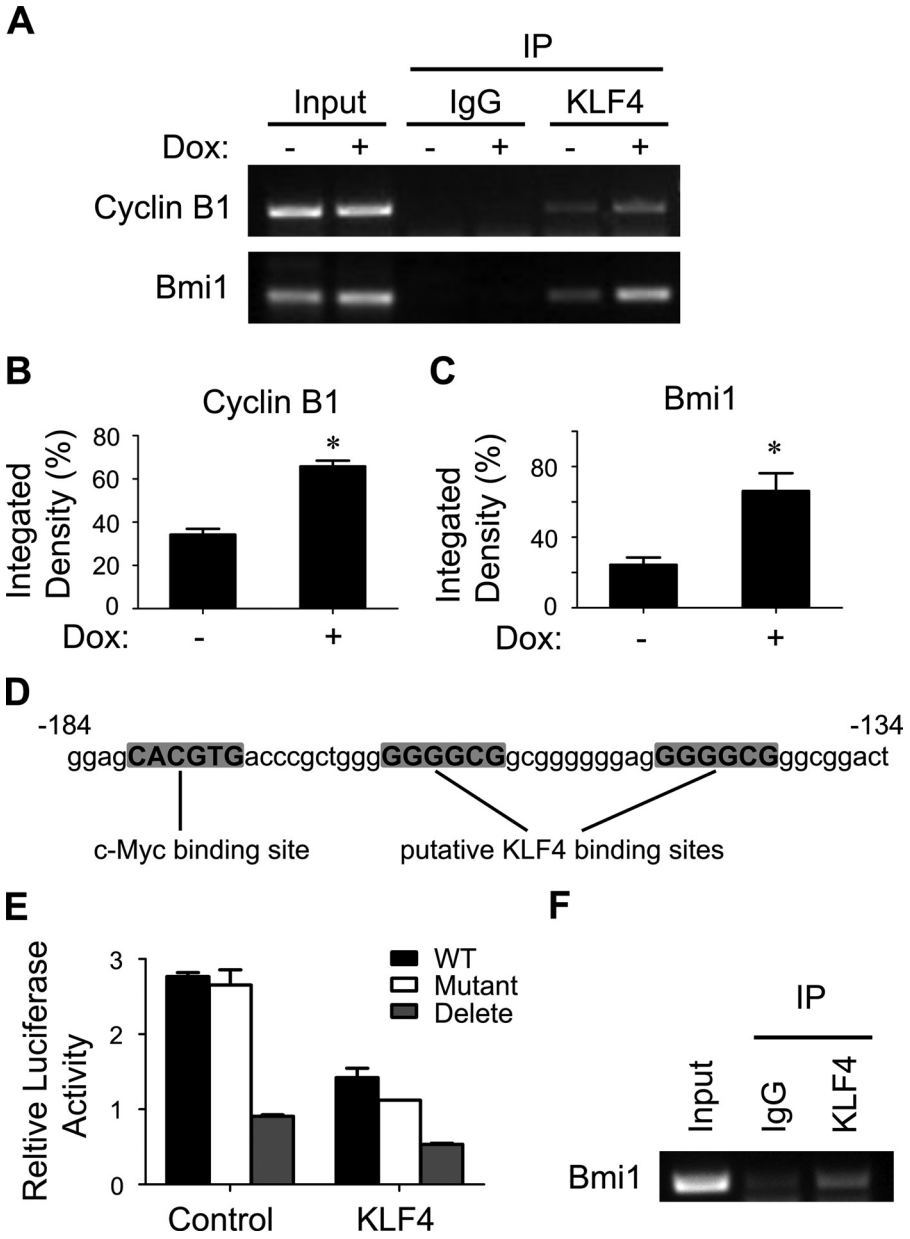


FIGURE 3. **KLF4 directly binds Bmi1 promoter and regulates Bmi1 expression and promoter activity in colon cancer cells.** *A*, ChIP assay testing interaction of KLF4 on Bmi1 promoter. IgG and KLF4 were used to precipitate chromatin DNA fragments as indicated; and DNA fragments from both Dox-induced (+) and non-induced (−) cells were used respectively. *B* and *C*, quantification of relative intensity of KLF4 binding with cyclin B1 (3B) and Bmi1 (3C) promoter, respectively. *D*, promoter region of Bmi1 gene with a c-Myc binding site and two putative KLF4 binding sites. *E*, luciferase reporter assay testing the effect of KLF4 on WT Bmi1 promoter, Bmi1 promoter with a mutation in c-Myc binding site (*Mutant*), or Bmi1 promoter deleted of two putative KLF4 binding sites (*Delete*) indicated in *D*. *F*, ChIP assay testing interaction between Flag-KLF4 and Bmi1 promoter, both of which were overexpressed in 293T cells by co-transfection. IgG and KLF4 were used to precipitate chromatin DNA fragments as indicated.

KLF4 Directly Binds Bmi1 Promoter and Regulates Bmi1 Expression in Colon Cancer Cells.—As a tumor suppressor protein in colon cancer (32), KLF4 crosstalks with Wnt/β-catenin and represses β-catenin-mediated gene expression (5). To test whether KLF4 regulates Bmi1 expression, we performed a reporter assay and demonstrated that Bmi1 promoter activity was suppressed by KLF4 in LS174T colon cancer cells (Fig. 2*E*). To analyze the effects of KLF4 on endogenous Bmi1 expression, we treated LS174T-KLF4 cells with doxycycline for 24 and 48 h to induce KLF4 expression. Bmi1 expression was examined by Western blot and semi-quantitative RT-PCR analysis. Both protein levels and RNA levels of Bmi1 were decreased upon KLF4 expression (Fig. 2, *F* and *G*). To confirm this result, we analyzed Bmi1 expression by real-time PCR. Again, the mRNA levels of Bmi1 were significantly decreased by KLF4 (Fig. 2*H*). Collectively, these data strongly suggest that KLF4 represses Bmi1 expression at transcription level in colon cancer cells.

To determine if KLF4 binds to Bmi1 promoter in colon cancer cells, we performed a ChIP assay using an anti-KLF4 antibody in LS174T-KLF4 cells. Cyclin B1, a known KLF4 target gene, was used as a positive control. PCR analysis with KLF4 specific primers demonstrated that KLF4 did bind to Bmi1 promoter, and the binding was increased upon doxycycline-induced KLF4 expression (Fig. 3*A*). To quantify the occupation of

KLF4 on Cyclin B1 and Bmi1 promoters, we analyzed the relative intensity of ChIP-PCR bands using Alpha Innotech AlphaView software. Integrated intensity values showed that the binding of KLF4 with both cyclin B1 promoter and Bmi1 promoter are significantly different between KLF4-induced and non-induced cells (Fig. 3, *B* and *C*).

The minimal essential binding site for KLF4 is 5′-G/AG/AGGC/TGC/T-3′ (33, 34). There is a c-Myc binding site and two putative sequences similar to KLF4 binding sequences in the Bmi1 promoter (Fig. 3*D*). Luciferase reporter assay was performed to test the effect of KLF4 on Bmi1 promoter with c-Myc binding site mutation or with deletion of the two putative KLF4 binding sites. Results showed no significant change in Bmi1 activity in response to KLF4 after mutation of c-Myc binding site, indicating that KLF4 inhibits Bmi1 independent of c-Myc. To our surprise, Bmi1 promoter depleted of the two putative KLF4 binding sites still responded to KLF4, suggesting that KLF4 inhibits Bmi1 by interacting with Bmi1 promoter but the direct interaction is not through these sites (Fig. 3*E*).

To test the specificity of KLF4 binding with Bmi1 promoter construct with deletion of the two putative KLF4 binding sites, ChIP assay was performed with 293T cells, which were co-transfected with Flag-KLF4 and Bmi1 promoter. Consistent with the luciferase assay, binding of KLF4 was still detected on the mutated Bmi1 promoter (Fig. 3*F*), suggesting that the GGGGCG sites are not required for KLF4 binding, and that the promoter sequence -233-0 is sufficient for KLF4 binding.

KLF4 Inhibits Bmi1-mediated Histone Ubiquitination—As a member of Polycomb group protein (PcG), Bmi1 is required for histone H2A ubiquitination and thus regulates gene silencing (22–24, 35). Knockdown of Bmi1 resulted in a decrease in H2A ubiquitination, which is consistent with previous reports (Fig. 4*A*). It has been reported that c-Myc regulates Bmi1 (30). As a control, c-Myc siRNA also decreased H2A ubiquitination. To test whether the inhibition of Bmi1 expression by KLF4 also leads to loss of H2A ubiquitination, we analyzed the level of ubiquitinated H2A in LS174T cells that express doxycycline-induced KLF4. Interestingly, we found that KLF4 expression significantly decreased the levels of ubiqutylated H2A while the total levels of H2A were not affected (Fig. 4*B*), suggesting KLF4 inhibits H2A ubiquitination, which is regulated by the Bmi1 complex.

To test whether Bmi1 overexpression can rescue cell proliferation repressed by KLF4, we established a stable cell line, LS174T-KLF4-Bmi1, which overexpresses Flag-tagged Bmi1 in addition to doxycycline-induced Flag-tagged-KLF4 (Fig. 4*C*). In the control cell line (LS174-KLF4), expression of KLF4 repressed Bmi1 expression (Fig. 4*C*, *left panel*). In LS174T-KLF4-Bmi1 cells, the expression of Bmi1 was not repressed by KLF4 (Fig. 4*C*, *right panel*).

Consistent with our previous report (5), expression of KLF4 inhibited the growth of LS174T cells (Fig. 4*D*). Bmi1 overexpression led to increase in growth rate of LS174T cells (Fig. 4*D*), indicating the role of Bmi1 in promoting colon cancer cell proliferation. This result is also consistent with the effects of our shRNA study on inhibiting colon cancer cell proliferation (Fig. 5, *A* and *B*).

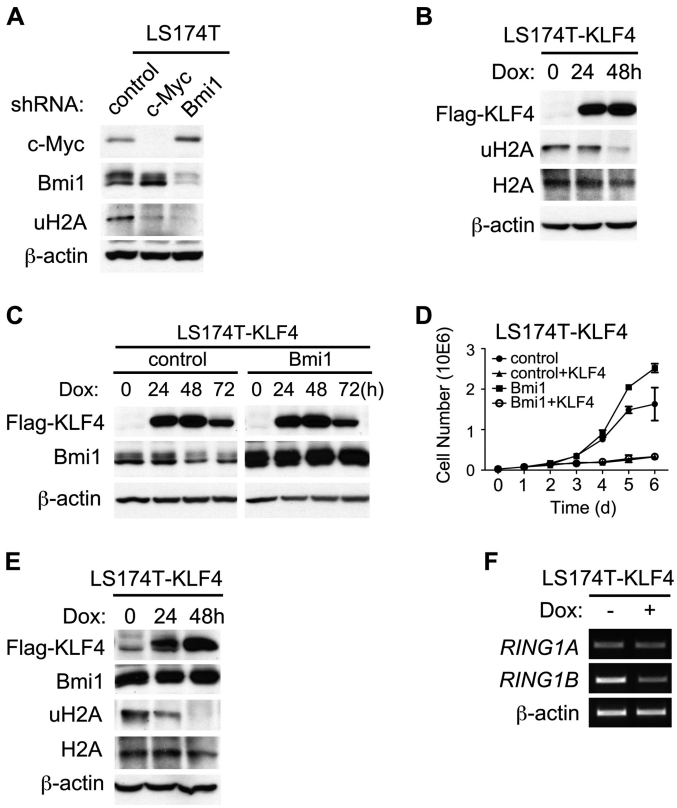


FIGURE 4. **KLF4 inhibits Bmi1-mediated H2A ubiquitination.** *A*, Western blot indicating expression of Bmi1 and ubiquitinated H2A (uH2A) in control and Bmi1 shRNA-expressing LS174T cells. *B*, level of uH2A in LS174T cells with Dox-induced KLF4 expression. *C*, Western blot showing expression of Bmi1 in LS174T cells expressing Dox-inducible KLF4, which were infected with control or Bmi1-carrying lentivirus, respectively. *D*, growth curves of LS174T cell lines that express Bmi1 and/or Dox-inducible KLF4 ($p < 0.0001$ between KLF4 *versus* Control, Bmi1 *versus* Control, and Bmi1+KLF4 *versus* Bmi1; $p = 0.7454$ between Bmi1+KLF4 *versus* KLF4). *E*, protein expression of Bmi1 and level of uH2A in the stable LS174T cell line under the combined effect of Bmi1 overexpression and inducible KLF4 expression. *F*, semi-quantitative RT-PCR testing transcription of other Polycomb complex members under the effect of Dox-induced KLF4 expression.

We found that Bmi1-mediated increase in cell proliferation was sequestered by KLF4; and KLF4-induced inhibition on cell proliferation was not rescued by Bmi1 expression (Fig. 4*D*). These results indicated that Bmi1 overexpression is not sufficient to rescue KLF4-mediated growth inhibition. We hypothesize that KLF4 regulates cell proliferation through multiple mechanisms.

To further test the mechanism how KLF4 regulates Bmi1-mediated H2A ubiquitination, we compared the ubiquitination status of H2A among KLF4-expressing cells, Bmi1-expressing cells and KLF4/Bmi1-double expressing cells. We found that Bmi1-mediated H2A ubiquitination can be attenuated by KLF4; however, KLF4-mediated inhibition of H2A ubiquitination cannot be rescued by Bmi1 overexpression (Fig. 4*E*). These results implicated that KLF4 inhibits H2A ubiquitination by more than one mechanism; other factors in the Bmi1 complex might also be regulated by KLF4.

To test this hypothesis, the mRNA levels of *RING1A* and *RING1B*, components of the Bmi1-polycom repressive complex, were analyzed by semi-quantitative RT-PCR. We found that the mRNA levels of *RING1B* but not *RING1A* were repressed by induced KLF4 expression in LS174T cells (Fig.

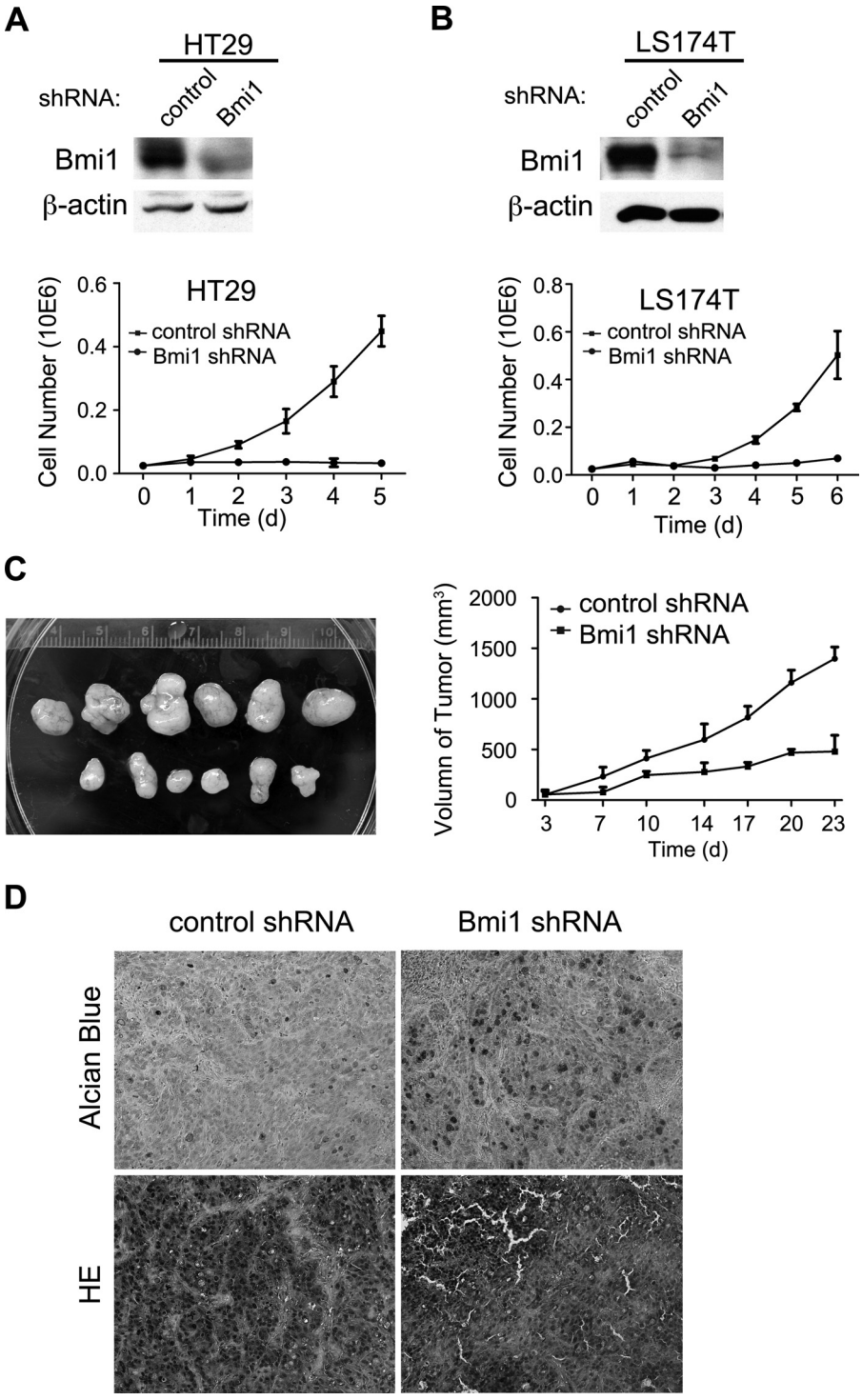


FIGURE 5. **Bmi1 is essential for colon cancer cell proliferation and tumor formation.** *A* and *B*, growth curves of the stable cell lines that express lentivirus-infected control/Bmi1 shRNA. Bmi1 expression levels were indicated in stable HT29 (*A*) and LS174T (*B*) cell lines with lentivirus-induced shRNA compared with control shRNA ($p < 0.0001$ between groups). *C*, *left*, tumors from control (*top*) and Bmi1 (*bottom*) shRNA xenograft mice 23 days after cell injection. *Right*, growth curve of tumors from control and Bmi1 shRNA xenograft mice ($p = 0.0002$ between groups). *D*, Alcian Blue (AB) and Hematoxylin and Eosin (HE) staining of tumor tissues from control and Bmi1 shRNA xenografts.

4*F*). It is of great interest to further study the mechanisms how KLF4 regulates H2A ubiquitination complexes as well as their functions in cell proliferation and tumorigenesis.

Bmi1 Is Essential for Colon Cancer Cell Proliferation in Vitro and in Vivo—Because Bmi1 acts as an oncogene that regulates cell proliferation and transformation of several types of cancers (16, 36), we tested whether Bmi1 also regulates proliferation of colon cancer cells. Bmi1 shRNA was transfected into different colon cancer cell lines through lentivirus infection. Cell proliferation assay indicated that Bmi1 down-regulation inhibited the growth of all tested colon cancer cell lines, including HT29 (Fig. 5*A*), LS174T cells (Fig. 5*B*) and KM20 (data not shown). To

determine the role of Bmi1 *in vivo*, we generated stable HT29 colon cell lines that express control shRNA or Bmi1 shRNA. These cells were injected subcutaneously into the flank of athymic nude mice. The tumors were measured twice a week for 3 weeks. We found that Bmi1 depletion significantly inhibited xenografted tumor growth (Fig. 5*C*), indicating that Bmi1 is essential for colon cancer progression.

The tumor sections were analyzed by H&E staining and Alcian Blue (AB) staining (Fig. 5*D*). The Bmi1 shRNA treated tumors had significant increase levels of AB stainings, which is a marker for glycoprotein mucin (Fig. 5*D*). AB staining is also used to identify goblet cells in the normal intestine and used as a marker for colon cancer cell differentiation (37). Our finding suggests that Bmi1 down-regulation inhibits tumor progression. Our previous work has demonstrated that KLF4 induced mucin expression in colon cancer xenografts (5). The increase in AB-positive cells in Bmi1 shRNA-treated colon cancer xenografts is consistent with our hypothesis that KLF4 represses Bmi1, inhibits proliferation, and regulates differentiation in colon cancer.

DISCUSSION

As a polycomb repressive protein, Bmi1 regulates a pool of genes and plays important roles in stem cell regulation and tumorigenesis. Activation of Wnt/β-catenin signaling is a hallmark of colorectal cancer; it interacts with many other signaling pathways in regulating both normal intestinal stem cells and cancer stem cells. In this study, we delineated the mechanisms of Bmi1 regulation in colon cancer cells. We found that Wnt/β-catenin signaling enhances Bmi1 transcription and KLF4 represses Bmi1 transcription. KLF4 also represses H2A ubiquitination by inhibiting the Bmi1 complex. Our findings suggest that Bmi1 is regulated by multiple mechanisms in colon cancer and is essential for colon carcinogenesis.

Bmi1 and Wnt/β-catenin signaling overlap roles in stem cell self-renewal, including hematopoietic stem cells (20) and intestinal stem cells (15), and are important links between stem cell and cancer (19). Wnt signaling regulates the expression of many stem cell markers, such as Lgr5 (14). Because Bmi1 is also a stem cell marker for the intestine, it is not surprising that Wnt/β-catenin signaling regulates the expression of Bmi1. Based on the TMA analysis of human colon cancer samples, the nuclear levels of β-catenin and Bmi1 have significant positive correlation in all tissue cores. Our results are consistent with the well-known function of Wnt/β-catenin signaling in colon caner and the role of Bmi1 as an oncogene in many tissues. However, there is no consensus β-catenin/TCF binding site in the promoter region of Bmi1 gene. We failed to detect the binding between β-catenin/TCF and the Bmi1 promoter by ChIP assay (not shown), suggesting that Wnt signaling indirectly regulates Bmi1 expression, probably through another β-catenin target. c-Myc is a well-known target of Wnt pathway (38). Within the Bmi1 promoter region, there is a c-Myc binding site and Bmi1 is a bona fide target of c-Myc oncoprotein (30). It is possible that Wnt/β-catenin signaling regulates Bmi1 through c-Myc, which is an important mediator of Wnt signaling in colon cancer (39).

In contrast, KLF4 directly binds the promoter of Bmi1 and represses Bmi1 expression. Although our finding from the ChIP assay suggests that the promoter sequence -233-0 is sufficient for KLF4 binding, we have not identified the direct binding sequence within this region. We cannot rule out the possibility that KLF4 indirectly binds -233-0 through another transcription factor or KLF4 binds additional site in Bmi1 promoter beyond this region. The role of KLF4 in stem cells and cancer is very complicated. KLF4 acts as a tumor suppressor in many cancers but may also act as a context-dependent oncogene (40). KLF4 inhibits cell proliferation and induces cell differentiation; however, it is one of the key factors required for iPS cell self-renewal.

KLF4 is down-regulated in most tumors but is also unregulated in a number of tumors, suggesting that the expression and function of KLF4 is dependent on the context of different tumors. For example, it has been suggested that the KLF4 acts as a tumor suppressor or oncogene depending on the status of p53, Ras, and $p21^{CIP1}$ (40). In the TMA study, the correlation between KLF4 and Bmi1 is not clear, because the level of KLF4 varies across grades 1, 2, and 3 in tumor tissues. KLF4 protein is most highly expressed in grade 3 of tumor tissues, probably because of additional genetic or epigenetic changes that altered KLF4 expression (supplemental Table S1).

Bmi1 is a member of the polycomb complex that plays important roles in chromatin remodeling and gene silencing. We found that KLF4 repressed both Bmi1 and *RING1B*, another member of this complex, and repressed H2A ubiquitination. We previously reported that KLF4 interacts with p300 and regulates histone acetylation (28). Regulating histone ubiquitination is novel function of KLF4. KLF4 acts as both transcriptional activator and repressor; it is not clear if Bmi1 can also act as a direct activator of transcription. It is important to further investigate the physiological roles of histone ubiquitination in cancer and stem cell biology.

shRNA knockdown experiments suggest that Bmi1 is required for colon cancer cell proliferation *in vitro* and *in vivo*. Interestingly, depletion of Bmi1 by shRNA not only inhibited cell growth, but also facilitated cell differentiation in xenograft tumors, as analyzed by mucin staining by AB. Mucin is a marker for goblet cells, which are regulated by KLF4 and Notch signaling. Inhibition of Notch signaling using a γ-secretase inhibitor resulted in goblet cell differentiation in adenomas of Apc^{Min} mice (37). We have shown that KLF4 induced goblet cell differentiation in colon cancer xenografts (5); this is consistent with the role of KLF4 in Bmi1 repression. The expression of KLF4 is also regulated by Notch pathway in the intestine (41, 42); it will be interesting to learn if Notch signaling interacts with Bmi1 in the intestine. The demonstrated role of Bmi1 in xenograft tumor growth is consistent with previous report that overexpressed KLF4 inhibited xenograft tumor growth (5) and with the finding that KLF4 inhibits Bmi1 as we discussed above.

Our findings demonstrate that Bmi1 is deregulated in colon cancer by multiple factors. Bmi1 could be used as a marker for colon cancer diagnostics. Our findings also demonstrate that Bmi1 is essential for colon cancer cell proliferation by regulating histone H2A ubiquitination. It is important to note that Bmi1 knock-out mice are viable (18, 43), suggesting that Bmi1 is

an ideal therapeutic target for human cancers, including colon cancer.

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